1. Complete microbiological examinations of the sludge and the fluidized bed microecosystem are required. These should be performed at start-up, before and after every significant system change, and at least monthly while the system is running. Samples of the fluidized bed should be taken from locations near both wall and center along the length of the bioreactor. In addition to gross inspection, samples should be subjected to examination by light and scanning electron microscopy. Lastly, standard laboratory analyses should be performed; see, for example, *Laboratory Procedures in Clinical Microbiology*, 2nd ed., J.A. Washington, ed. Springer-Verlag: New York, 1985.

2. Establishment of a stable inoculum is essential for the success of this project. This will include purification of the active, i.e., perchlorate- and nitrate-reducing genera, species, strains, etc. It will be important to determine whether the active organisms produce any undesirable materials, toxins, etc. Presumably, the organisms involved are rather benign since they originate from food-processing; however, selective metabolism (and elimination of competition) may effect evolution of undesirable variants. Once a purified inoculum is available, it will be desirable to develop a fast, easy, straightforward laboratory test—preferably an immunoassay—for routine assessment of the microorganisms. Moreover, it may be possible to preferentially select for organisms that favor perchlorate and nitrate over oxygen. Natural evolution itself should eventually lead this way if organisms are continuously exposed to water where $[O_2]$ is very low relative to the concentrations of reducible oxyanions.

3. Samples of the fluidized bed and the purified inoculum should be submitted for wet ashing with a subsequent determination of transition metal content by atomic absorption, atomic emission, and/or inductively-coupled plasma spectrophotometry. If graphite-furnace sample atomization is used, wet ashing is unnecessary. Analyses should focus on transition metals that possibly act in the catalytic reduction of perchlorate and nitrate; these would include molybdenum and titanium in addition to other more commonly encountered metals: copper, lead, iron, cobalt, manganese, chromium, nickel, and zinc. The crucial metal(s) must be naturally present in the raw water in sufficient quantities; otherwise, there would be no growth.

4. When the microbial ecosystem goes awry, and the growth of ineffective organisms is observed, these should be collected for examination and analysis to determine whether they are fungi, protistans, or monerans. In addition, an effort should be made to determine whether they compete with the desired organisms for food, feed on the desired organisms, or otherwise adversely affect the system as opposed to being an artifact of a corrupted system. In other words, it is imperative to determine whether these organisms are the cause of the problem or its effect. It is even possible that these masses contain agglutinated cells of the desired organism.

5. Although Phase 1 assessed a number of water quality parameters, most of the effort was directed toward ensuring that contaminants present in the sourcewater, e.g., VOCs, were removed. Very little effort was expended in ascertaining whether the oxyanion reduction process contributed any new materials into the water. We would expect a mixture of biomolecules to be released. Undoubtedly, some cells will be lysed, and their contents will spill into the water. Fragments of peptidoglycans, proteins, polysaccharides, and all of the intermediates of cellular metabolism can be expected. That is why it is so important to measure total organic carbon (TOC) concentration and dissolved organic carbon (DOC). These two measurements provide a firsthand estimate of material being contributed to the water by the organisms.

6. § 3.0, pg. 3, col. 2, ¶ 3: It is stated that: "... the microorganisms multiply to a steady-state level, determined by the organic loading to the system." It is unclear exactly what is meant by the term *steady-state level.* Phase 1 showed that growth and reproduction continued seemingly without end; i.e., the rate of death was exceeded by the rate of reproduction, hence the need for the growth controller that was installed. Moreover, growth and reproduction cannot be limited simply by lowering the amount of food added. That food is necessary as a reductant to react with the oxidants: perchlorate, nitrate, or oxygen. If insufficient food is added, the microbes will be unable to reduce the perchlorate. The determining factor is the total number of electrons needed to react with the total reducible (oxidizing) capacity of a varying mixture of O_2, ClO_4^-, and NO_3^-. Bacteria are not like animals. If bacteria are alive, they are—for all intents and purposes—reproducing. A human can spend 90 years taking in food and oxygen without reproducing; that is the type of behavior I think the contractor is alluding to. This is not generally true for bacteria and most protistans. If a bacterium is taking in nutrients and oxidants, it is working towards reproduction. Accordingly, there is no steady state until the death rate equals the reproduction rate.

7. § 3.0, pg. 3, col. 2, ¶ 4: Sentence 1 states that nonviable (dead and dying?) microorganisms eventually become detached from the medium and exit the system in the effluent, allowing new microorganisms to attach and reproduce. This is an oversimplification, possibly even wrong. Phase 1 never demonstrated (according to the documents received) that the microbes detached from the fluidized bed. It is possible that (1) new organisms attach to the dead ones on the surface, much as cocci can attach to one another, occasionally breaking off, (2) the available surface area of the carbon is so large that only a small amount of the total surface was ever colonized and covered in Phase 1, (3) that live organisms continually vie for places on the substrate displacing one another as they reproduce, or (4) dead ones fall off and live ones take their place as is suggested by the contractor.

   However, it seems most likely that the organisms attach to the substrate via polysaccharides embedded in the cell walls. As such, all of these scenarios probably occur

to some degree. It also seems likely that the microbes, if they are in fact bacteria, reproduce by conjugation and binary fission; consequently, reproduction probably occurs right on the substrate with new organisms already tethered to the surface at their inception. Some of these possibilities could be ruled out—if not confirmed—by optical microscopic examination of carbon granules.

8. § 3.0, pg. 3, col. 2, ¶ 4: Sentence 2 states that "The reaction takes place under anoxic conditions; therefore, no air or oxygen (other than that contained in the influent water) is introduced to the system." If current understanding is correct, once perchlorate is reduced to chlorite, a dismutase effect the disproportionation to molecular dioxygen and chloride. In this case, the organisms would presumably then consume this dioxygen, since they appear to prefer it to perchlorate as an oxidant. Strictly speaking, it is probably not true that the system is anoxic. The water itself probably remains very low in oxygen concentration. Even if some oxygen permeates the membrane of an individual cell, a neighboring cell could then consume it. An additional point: The logic of the sentence construction implies a cause and effect relationship, but none exists. The fact that the reaction takes place without oxygen does not then mean that no air or oxygen is introduced to the system. Rather, the fact that cellular metabolism produces very little *extracellular* oxygen (if any) leads to this conclusion, but only as long as there are no mechanical contributions (e.g., a leak).

9. § 3.2, pg. 4, col. 2, ¶ 1 (cont. from col. 1): Full sentence 2 states that "... the biomass will be 15 feet high." I assume that this refers to the height of the fluidized bed, much of which is actually suspended carbon granules. I have seen no evidence of any attempt to measure the thickness of the fixed film on the carbon surface. I suppose it could probably be done by light or electron microscopy, and it may even be useful in evaluating surface adhesion. This point requires clarification.

10. § 3.2, pg. 4, col. 2, ¶ 1: If the size of the particulate cellular debris is unknown, how can the effectiveness of the sand filters be ensured? What surrogates, if any, will be used to test the sand filters?

11. § 4.2, pg. 5, col. 1, ¶ 1: The discussion of surrogates requires expansion. What sort of strategy will be used? Is the plan to introduce a surrogate chemical species that is more easily determined? Or is it to link another property with successful perchlorate reduction? If the plan is a chemical surrogate, the services of an inorganic chemist are absolutely necessary. Whatever the plan is, EPA approval of the surrogate must be a condition of the work plan.

12. § 4.2, pg. 5: Regarding the establishment of system operating parameters

    a. Col. 1, ¶ 1: Sentence 2 states that no real-time instrumentation is available for measurement of perchlorate. Strictly speaking, this is true; however, ion-selective electrodes are available that can fairly reliably semi-quantitatatively determine perchlorate concentrations down to 10^{-6} mol dm^{-3}, about 10× the California action level of 18 ng mL^{-1} = 0.19 μmol dm^{-3}. While the electrodes suffer from some interferences, the primary ones are from nitrate and chlorate. If nothing else, ISEs in flow-through diverter loops could serve as gross indicators of system performance (especially failure). If the ISE shows a high perchlorate concentration, clearly there is a malfunction.

    The contractor should perform (or subcontract) a laboratory bench scale evaluation of ISEs for nitrate and perchlorate to verify the linear dynamic range and the lower limit of quantitation. If this proves reasonably fruitful, nitrate-selective and perchlorate-selective electrodes could be used alongside pH electrodes (the most common ISEs) in a diverter loop as suggested above.

    b. Col. 1, ¶ 1: Final sentence has the Roman letter "u" rather than the Greek letter "μ" to represent micrograms. Correct.

    c. Col. 2, ¶ 1: Sentence continuing from col. 1 has lowercase "v" as abbreviation for volt. Correct by replacing with capital "V."

13. § 4.3, col. 1, ¶ 1: Sentence continued from previous page has the word "inoculum" misspelled. Delete the extra letter "n."

14. § 5.0, pg. 6, col. 2, ¶ 3, line 9: There is an extra space after the word "will."

15. § 5.0, pg. 7: Regarding the "Treatment System Description"

    a. There is no guarantee that thte hydrodynamics of a larger reactor will favor the same microorganisms. Since a microbiological study was not done, it is impossible to say. The new reactor may suffer from byproducts that were not found in the Phase 1 bioreactor.

    b. It would be better to test other food sources than ethanol, e.g., corn syrup. The flammability of ethanol and the need for a permit from the Bureau of Alcohol, Tobacco, and Firearms make this a suboptimal food source. A cheap nonflammable nutrient would be ideal.

c. Given the size of the Phase 2 bioreactor, it would be better to have multiple ports (at the same height) along the length of the fluidized bed chamber. There is no guarantee that flow will be sufficiently turbulent to provide good mixing. This sampling error may lead to false high or low values. This will allow for some averaging out of sampling errors. It will also indicate where dead spots occur in the bioreactor.

d. Scale drawings of the bioreactor and components should be submitted for EPA review and approval before any plans go to a subcontractor for construction. Hard copies of the designs should be listed as deliverables, and a firm date should be set for receipt by EPA.

e. There needs to be a mechanism for sampling deep within the carbon bed, as opposed to flow near the bioreactor wall.

16. § 9.0, pgs. 9-10: Regarding the "Pilot System Operation and Maintenance Plan"

a. A two-week start-up time is probably insufficient; a period of 3-4 weeks is more reasonable.

b. The system should be run with ground water before any carbon is added, and sampling should be done at three additional times: (1) before the carbon is added, (2) after carbon is added, but before inoculation, and (3) immediately after inoculation.

c. Since both the identities of the microorganisms and their waste products are unknown, running the system as a closed loop (during start-up) may actually be poisoning the organisms with their own waste products. This must be considered. For example, likely by-products of ethanol metabolism are ethanal and ethanoic acid; these (especially ethanal) may be toxic.

d. Temperature and pH should be monitored on-line with ports for flow-compatible electrodes or, better yet, a diverted flow loop. Obviously, these must be located near the sampling ports. There is no reason that pH and temperature cannot be followed essentially continuously, with automated data collection (recording) at a rate of one point an hour. The software could be programmed specifically to record any spikes.

e. The inoculating process needs to be described in detail. Right now, there is no detail. That is entirely unsatisfactory.

f. Section 9 consists almost entirely of § 9.1, and § 9.2 is unrelated. There is insufficient breakdown (in terms of outlining) of the process to be used in setting up the system. In terms of the science, this is one of the most important sections, and yet it has less real detail than the permitting section (§ 8.0). I realize that it is easier and more straightforward to write the permitting section; nonetheless, it is important to describe as fully as possible exactly what is to be done. Good science requires good planning. A sense of "we haven't quite figured out how we're going to do this, so we'll leave it up in the air for now" pervades this section; it is too vague. Since it is relatively unrelated, § 9.2 should be moved to elsewhere.

17. § 10.0, pgs. 10-11: Regarding the "Sampling and Analysis Plan"

a. Col. 2, ¶ 1: If the identity of the active microorganism(s) is(are) not known, which is presently the case, it is impossible to determine if said microbes are receiving sufficient organic substrate and nutrients. This performance-based assessment was criticized in the report on Phase 1 as well. The contractor does not know what trace metals the microbes need, for example. The work plan should refer to a list of specific analytes; presumably, these are included in the list in § 10.3, pg. 11, ¶ 1, sentence 1. This requires clarification.

b. Col. 2, ¶ 2: Sentence 2 has the word "inoculum" misspelled. Delete the extra letter "n."

c. Col. 2, ¶ 2: What is meant by sentence 2? Define "characterization." I hope that this suggests examination, analysis, and evaluation to the level of rigor and sophistication I have suggested in this review and the review of the Phase 1 report, but I suspect it does not. This subsection is too vague. The input of a microbiologist and/or bacteriologist is required.

18. § 10.2, pg. 11, col. 1: It is unnecessary to repeat the sample port locations, which were already listed in § 5.0, pg. 7, col. 2. A figure would be far better as explained elsewhere in this review.

19. § 10.3, pg. 11, col. 2, ¶ 1: Total and dissolved organic carbon concentrations should be determined by thermally promoted peroxydisulfuric acid wet ashing and by high temperature combustion analysis. These were excluded from the list in Phase 1; this mistake should be rectified in Phase 2.

It is debatable whether there is any benefit to analyzing for other organic analytes, e.g., carboxaldehydes (incomplete oxidation products) or α-oxocarboxylates (intermediates in the citric acid cycle) as the pentafluorobenzyloximes by GC-ECD. At this point, such extensive and intensive analyses are probably premature.

I do have some reservations about using an unidentified microorganism, even if it did come from the food-processing industry. One never knows if such an organism could possess an endotoxin that would be released as cells are lysed. If this be the case, then the sand filters may act as toxin reservoirs until backwashed. In the case of exotoxins, these are generally sufficiently degradable so as not to survive a subsequent disinfection process. If a bacteria-based bioreactor is run for long periods without complete breakdown and sterilization, one has to consider the possibility of a lysogenic phage infecting the cells and contributing a gene that promotes toxin formation. This is another good reason for clearly identifying, isolating, and studying the active organism. (Cf. comment 2.)

20. § 10.3, pg. 11, col. 2, ¶ 2: The specific methods should be cited by number and date. Any non-EPA methods should be fully specified as standard journal or reference book citations. Complete citations for all methods to be used should appear in the references, § 13.0.

21. § 12.0, pg. 12, col. 2: The schedule is overly ambitious. It appears that the contractor is anxious to get to what is viewed as the meat of the project without spending sufficient time and effort on supporting and background research and development. While I can appreciate the results-oriented approach, the results will not be especially useful or applicable if we don't have a good grasp of what is going on at a fundamental level. Also, we won't be able to fix the system if it breaks down without a thorough knowledge of the basics. This was also discussed in the review of the Phase 1 report.

22. § 13.0, pgs. 12-13: Regarding the references. Complete citations for all laboratory analysis techniques or methods to be used should appear in the references. (Cf. comment 20.)

23. Generally speaking, it seems that the contractor's staff is deficient in expertise in microbiology, ecology, bacteriology, mycology, cell biology, bioinorganic chemistry, and traditional biochemistry. From the content and emphasis of the reports and work plans, it appears that the HLA staff is a mixture of civil, environmental, and mechanical engineers. Nearly all of the detail for this study—in either phase—is engineering in nature. The overall study design would be improved by the addition of several experts whose collective expertise encompasses the areas listed above. The strategy for some of this work seems rather amateurish and unscientific, especially where the disciplines listed above are concerned.

24. Work on a number of these areas could begin immediately. It need not wait until the main thrust of Phase 2 gets underway. The first thing HLA should do is hire a bacteriologist, a general microbiologist, and a bioinorganic chemist. ▫